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# Nitric Oxide Decreases Cytokine-induced Endothelial Activation

## Nitric Oxide Selectively Reduces Endothelial Expression of Adhesion Molecules and Proinflammatory Cytokines

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### Abstract

To test the hypothesis that nitric oxide (NO) limits endothelial activation, we treated cytokine-stimulated human saphenous vein endothelial cells with several NO donors and assessed their effects on the inducible expression of vascular cell adhesion molecule-1 (VCAM-1). In a concentration-dependent manner, NO inhibited interleukin (IL)-1 $\alpha$ -stimulated VCAM-1 expression by 35–55% as determined by cell surface enzyme immunoassays and flow cytometry. This inhibition was paralleled by reduced monocyte adhesion to endothelial monolayers in nonstatic assays, was unaffected by cGMP analogues, and was quantitatively similar after stimulation by either IL-1 $\alpha$ , IL-1 $\beta$ , IL-4, tumor necrosis factor (TNF $\alpha$ ), or bacterial lipopolysaccharide. NO also decreased the endothelial expression of other leukocyte adhesion molecules (E-selectin and to a lesser extent, intercellular adhesion molecule-1) and secreted cytokines (IL-6 and IL-8). Inhibition of endogenous NO production by L-N-monomethyl-arginine also induced the expression of VCAM-1, but did not augment cytokine-induced VCAM-1 expression. Nuclear run-on assays, transfection studies using various VCAM-1 promoter reporter gene constructs, and electrophoretic mobility shift assays indicated that NO represses VCAM-1 gene transcription, in part, by inhibiting NF- $\kappa$ B. We propose that NO's ability to limit endothelial activation and inhibit monocyte adhesion may contribute to some of its antiatherogenic and antiinflammatory properties within the vessel wall. (*J. Clin. Invest.* 1995; 96:60–68.) **Key words:** nitric oxide • endothelium • adhesion molecules • monocytes • atherogenesis

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### Introduction

Nitric oxide (NO)<sup>1</sup> or closely related molecules account for the activity of endothelium-derived relaxing factor (1, 2). This mediator stimulates guanylyl cyclase in smooth muscle cells leading to vascular relaxation (3). Besides its vasodilatory effects, NO reportedly has many antiatherogenic properties. NO reduces platelet aggregability (4), limits vascular smooth muscle cell proliferation (5, 6), inhibits leukocyte adhesion to the endothelium (7, 8), and prevents monocyte chemotaxis (9). Chronic provision of L-arginine in the diets of hypercholesterolemic rabbits has been reported to improve endothelium-dependent vasodilation and to reduce the extent of atherosclerotic lesions (10). Local leukocyte recruitment to the vessel wall is an early step in atherogenesis (11), and endothelial-leukocyte adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and endothelial-leukocyte adhesion molecule-1 (ELAM-1 or E-selectin) may facilitate recruitment of monocytes and lymphocytes to sites of lesion formation (12–15). Therefore, factors which modulate the expression of endothelial-leukocyte adhesion molecules and other endothelial-derived cytokines may be important in modulating lesion formation. This study sought to determine whether NO can limit endothelial activation by inhibiting adhesion molecule expression and monocyte adhesion.

### Methods

**Reagents.** Sodium nitroprusside (SNP) was purchased from Elkins-Sinn (Cherry Hill, NJ), and 3-morpholino sydnonimine (SIN-1) was a gift from Dr. A. G. Cassella (Frankfurt, Germany). S-nitroso-glutathione (GSNO) was chemically synthesized from glutathione and sodium nitrite (Sigma Immunochemicals, St. Louis, MO) (16). IL-1 $\alpha$  was obtained from Hoffmann-La Roche (Nutley, NJ). IL-1 $\beta$ , TNF $\alpha$ , and IL-4 were purchased from Genzyme Corp. (Cambridge, MA). *Escherichia coli* lipopolysaccharide (LPS) was purchased from Sigma Immunochemicals. Cell-permeable superoxide dismutase (SOD) and catalase coupled to polyethylene glycol (PEG) were obtained from Sigma/Enzon (St. Louis, MO) (17). Assays for IL-6 and IL-8 were measured according to commercially available enzyme immunoassay (EIA) kits (R & D Systems, Inc., Minneapolis, MN), assayed directly in human saphenous vein endothelial cell (HSVEC) supernates.

**1. Abbreviations used in this paper:** CAT, chloramphenicol acetyltransferase; EIA, enzyme immunoassay; GSNO, S-nitroso-glutathione; hpf, high-power field; HSVEC, human saphenous vein endothelial cells; ICAM-1, intercellular adhesion molecule-1; L-NAME, L-nitro-arginine methyl ester; L-NMA, L-N-monomethyl-arginine; NO, nitric oxide; SIN-1, 3-morpholino sydnonimine; SNP, sodium nitroprusside; VCAM-1, vascular cell adhesion molecule-1.

**Cell culture.** HSVEC were harvested enzymatically with type II collagenase 0.1% as described (18) and maintained in medium 199 (GIBCO BRL, Gaithersburg, MD), containing Hepes (25 mmol/liter), heparin (1%), endothelial cell growth factor (50  $\mu$ g/ml), L-glutamine (1%), antibiotics, and 5% fetal calf serum (Hyclone Laboratories, Logan, UT). Once grown to confluence, the cells were replated on low pyrogen fibronectin (1.5  $\mu$ g/cm<sup>2</sup>) at 20,000 cells/cm<sup>2</sup>. HSVEC isolated by these techniques form a confluent monolayer of polygonal cells and express von Willebrand factor as determined by their content of specific mRNA and immunoreactive protein (18). Cell number was assessed after trypsinization in a Neubauer hemocytometer (VWR Scientific Corp., Philadelphia, PA). Cellular viability was assessed by Trypan blue exclusion. Bovine aortic endothelial cells (10<sup>6</sup>/100-mm culture dish, grown in DME with 5% heat-inactivated fetal calf serum) were used within passage 3 for transfection experiments.

**Detection of cell surface molecules.** Assay of cell surface molecules was carried out after treatment with cytokines and NO donors for 24 h. The expression of adhesion molecules was determined by cell surface EIA or flow cytometry using mouse anti-human monoclonal antibodies against VCAM-1 (antibody E1/6), E-selectin (Ab H18/7), ICAM-1 (Ab HU 5/3), MHC-I (W6/32) (19), or a constitutive and non-cytokine-inducible endothelial cell antigen (E1/1) (20). These last two endothelial surface molecules share a high level of basal constitutive expression, although MHC-I antigen (Ag), at variance from E1/1 Ag, shows some degree of inducibility by cytokines. EIA were carried out by incubating monolayers first with saturating concentrations of specific monoclonal antibodies against the target molecule, then with biotinylated goat anti-mouse IgG (Vector Labs, Inc., Burlingame, CA), and finally with streptavidin-alkaline phosphatase (Zymed Laboratories, Inc., South San Francisco, CA). Layers were washed three times between each incubation step, and integrity of the monolayer was monitored by phase-contrast microscopy. The surface expression of each protein was quantified spectrophotometrically, reading the optical density of the wells (410 nm) 15–60 min after the addition of a chromogenic substrate (para-nitrophenylphosphate), as described (19).

Flow cytometric analysis of adhesion molecules was performed by suspending HSVEC in Hanks' buffered saline solution containing 3 mmol/liter EDTA and incubating the cells with specific primary antibody for 30 min at 4°C. After washing, the cells are then exposed to goat anti-mouse F(ab')<sub>2</sub> labeled with fluorescein-isothiocyanate (Caltag Laboratories, San Francisco, CA) at 4°C. After further washing, the cell suspension was passed through a FACScan® analyzer (Becton-Dickinson Immunocytometry Systems, Mountain View, CA). Results were plotted as intensity of fluorescence (arbitrary units, on logarithmic scale, on the abscissa) versus cell number (on the ordinate, total cells counted, 10<sup>4</sup> for each condition).

**Assessment of total protein synthesis.** HSVEC were cultured in 96-well plates and stimulated with IL-1 $\alpha$  in the presence and absence of NO donors. After 24 h, the total cell-associated protein content was assessed by the amido black assay, as previously described (21). Briefly, HSVEC monolayers were fixed with 4% formaldehyde in a 0.1 mol/liter acetate buffer, pH 3.1, and incubated with amido black B (Sigma Immunochemicals) to stain cell proteins (22). Unbound dye was rinsed away with repeated PBS washings, and dye uptake was determined at 620 nm in an EIA microtiter photometer. In parallel experiments, dye binding correlated linearly ( $r = 0.98$ ) with cell number per well, which in turn was determined by hemocytometric counting of trypsinized cells in parallel cultures.

**Preparation of human monocytes and U937 cells and adhesion assays.** Human peripheral blood monocytes were obtained by centrifugation of white blood cell-enriched blood (Dana Farber Cancer Institute, Boston, MA) on Ficoll-Hypaque density gradient at 15°C (LMS; Organon Teknika, Durham, NC) followed by counterflow centrifugation elutriation in a Beckman J2–21 M/E centrifuge using a JE-6 elutriation rotor and a 6-ml Sanderson chamber (Beckman Instruments, Inc., Palo Alto, CA). A modified Doherty's method was used (23, 24): the elutriation buffer was Dulbecco's modified phosphate-buffered saline (GIBCO BRL), supplemented with 3 mmol/liter EDTA and 0.25% human serum

albumin (HSA; Sigma Immunochemicals). The mononuclear cell fraction isolated by Ficoll-Hypaque density gradient at the Ficoll-plasma interface was loaded at 14 ml/min onto the elutriator centrifuge rotor head (2,500 $\pm$ 10 rpm at 10°C), and fractions of elutriated cells were collected. Monocytes were eluted at 21.5 ml/min. Monocyte suspensions with this technique are 89 $\pm$ 4% pure with 8% lymphocyte, < 2% granulocyte, and essentially no platelet contamination as determined by light scatter (FACScan®; Becton Dickinson Immunocytometry Systems) and cell surface antigen analysis with mAb directed to CD14. Monocytes were resuspended in cold Dulbecco's modified phosphate-buffered saline containing 0.75 mmol/liter Ca<sup>2+</sup> and 0.75 mmol/liter Mg<sup>2+</sup> and 0.2% HSA. U937 cells were obtained through American Tissue Culture Collection (Rockville, MD) and grown in RPMI 1640 (GIBCO BRL) containing 10% fetal calf serum. Both monocytes and U937 cells were concentrated by centrifugation at 1  $\times$  10<sup>6</sup> cells/ml.

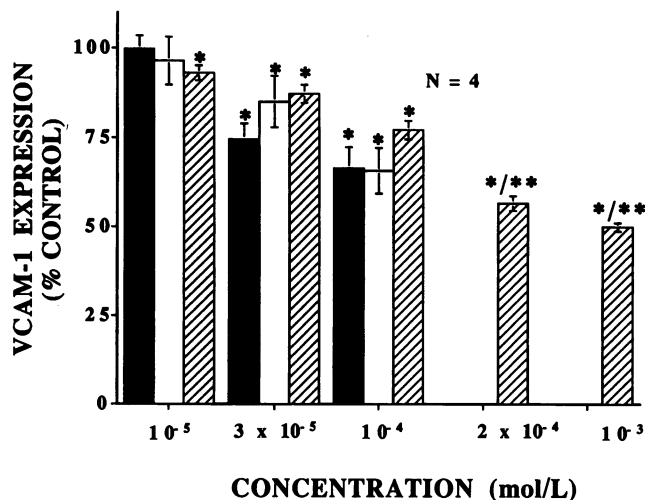
For the adhesion assays, HSVEC were grown to confluency in 6-well tissue culture plates, after which IL-4 (50 ng/ml) or IL-1 $\alpha$  (10 ng/ml) was added for an additional 24 h, in order to induce the expression of VCAM-1, in the presence or absence of GSNO (0.2  $\times$  10<sup>-3</sup> mol/liter). For control, some monolayers were treated with a mouse anti-human monoclonal antibody (E1/6) against VCAM-1. The adhesion assay was performed by adding 1 ml of the concentrated monocytes or U937 cell suspension to each monolayer under rotating conditions (63 rpm) at 21°C. After 10 min, nonadherent cells were removed by gentle washing with medium 199, and monolayers were fixed with 1% paraformaldehyde. The number of adherent cells was determined by counting six different high-power fields (hpf) using an ocular grid and a  $\times$  20 objective (0.16 mm<sup>2</sup>/field). Fields for counting adherent leukocytes were randomly located at half-radius distance from the center of the monolayers.

**Northern analysis.** HSVEC RNA was isolated by ultracentrifugation through CsCl (25). Total RNA (20  $\mu$ g) was separated on a 1.2% agarose-formaldehyde gel, transferred to a nylon membrane (Hybond-N; Amersham Corp., Arlington Heights, IL) and immobilized by shortwave ultraviolet illumination. cDNA probes were labeled by random priming, [<sup>32</sup>P]CTP (3,000 Ci/mmol), and Klenow fragment of DNA polymerase I (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ). All blots were hybridized at 42°C overnight and washed (0.2  $\times$  SSC, 0.1% SDS, 63°C) before autoradiography at -80°C for 24–72 h.

**Nuclear run-on assay.** Nuclei from 3–5  $\times$  10<sup>7</sup> endothelial cells were prepared, and in vitro transcription with [ $\alpha$ -<sup>32</sup>P]UTP (800 Ci/mmol) was performed as described (26). Linearized plasmids (1  $\mu$ g) were immobilized on nylon membranes, hybridized to radiolabeled transcripts ( $\sim$  5–8  $\times$  10<sup>7</sup> cpm/ml) at 45°C for 48 h in hybridization buffer containing 50% formamide, 5  $\times$  SSC, 2.5  $\times$  Denhardt's Solution, 25 mM sodium phosphate buffer (pH 6.5), 0.1% SDS, and 250 mg/ml salmon sperm DNA, and washed in 1  $\times$  SSC/0.1% SDS at 65°C before autoradiography.

**Transfections.** The human VCAM-1 promoter constructs containing the chloramphenicol acetyltransferase (CAT) reporter were described previously by Neish et al. (27). [-755]F0.CAT is the functional VCAM-1 promoter containing AP-1, GATA, and NF- $\kappa$ B binding sites. [-98]F3.CAT contains the NF- $\kappa$ B binding sites without GATA or AP-1. [-44]F4.CAT lacks NF- $\kappa$ B binding sites. Bovine aortic endothelial cells were transfected with each reporter plasmid (25  $\mu$ g) using the calcium phosphate precipitation method (28). As an internal control for transfection efficiency, pRSV. $\beta$ GAL plasmid (10  $\mu$ g) was cotransfected in all experiments. Preliminary results using  $\beta$ -galactosidase staining indicate that cellular transfection efficiency was  $\sim$  12–15%. Cells (60–70% confluent) were stimulated 48 h after transfection with IL-1 $\alpha$  (10 ng/ml) and TNF $\alpha$  (10 ng/ml) (alone or in combination with GSNO [0.2 mmol/liter]), and cellular extracts were prepared 12 h later using lysis buffer (100 mg/ml leupeptin, 50  $\mu$ g/ml aprotinin, 0.1 mM PMSF, 5 mM EDTA, 5 mM EGTA, 100 mM NaCl, 5 mM Tris-HCl, pH 7.4) and one freeze-thaw cycle. The cellular extracts were centrifuged at 12,000 g for 10 min, and the supernatant was subjected to CAT and  $\beta$ -galactosidase assay.

**CAT assays.** CAT activity was determined by incubating the super-



**Figure 1.** The effect of SNP (black bars), SIN-1 (white bars), and GSNO (hatched bars) on IL-1 $\alpha$  (10 ng/ml)-stimulated VCAM-1 expression. IL-1 $\alpha$  and NO donors were coincubated for 24 h before cell surface EIA. Results are expressed relative to absence of NO donors (percentage of control). Original readings were derived from four different experiments each consisting of  $\geq 12$  different replicates for each condition. \* Significant difference ( $P < 0.05$ ) compared with controls and \*\*significant difference ( $P < 0.01$ ) compared with the effects of SNP and SIN-1 at 0.4 mol/liter.

nantant (100  $\mu$ l) with [ $^3$ H]chloramphenicol (50  $\mu$ Ci/ml) and *n*-butyryl coenzyme A (250  $\mu$ g/ml) for 20 h at 37°C (29). The *n*-butyryl [ $^3$ H]-chloramphenicol was then separated from unmodified chloramphenicol by xylene phase extraction and counted for 2 min in a liquid scintillation counter (LS1800; Beckman Instruments, Inc.). CAT activity was calculated from a standard curve using various concentrations of purified CAT (Promega, Madison, WI).  $\beta$ -Galactosidase activity was assayed spectrophotometrically (absorption at 410 nm) and compared with a standard curve using known amounts of purified  $\beta$ -galactosidase (Sigma Immunochemicals) as described previously (30). The normalized CAT activity was calculated as the ratio of CAT to  $\beta$ -galactosidase activity. The relative CAT activity was standardized to the normalized F0.CAT activity having a value of 100. Each experiment was performed three times in duplicate and all experiments included both positive (highly expressed pSV40.CAT) and negative (promoterless po.CAT) controls.

**Electrophoretic mobility shift assay.** Nuclear extracts were prepared as described (31). The NF- $\kappa$ B oligonucleotide (CCTGGGTTTCCC-CTTGAAGGGATTTCCTCC) spanning the two tandem  $\kappa$ B sites in the human VCAM-1 promoter (27) was synthesized by Genosys Biotechnologies, Inc. (The Woodlands, TX). The oligonucleotide was end-labeled by T4 polynucleotide kinase (New England Biolabs Inc., Beverly, MA) and [ $\gamma$ - $^{32}$ P]ATP (3,000 Ci/mmol) and purified by Sephadex G-50 columns (Pharmacia LKB Biotechnology, Inc.). Nuclear extract (10  $\mu$ g) was added to  $^{32}$ P-labeled NF- $\kappa$ B oligonucleotide (~20,000 cpm) in a buffer containing 2  $\mu$ g poly[dI·dC] (Boehringer Mannheim Corp., Indianapolis, IN), 10  $\mu$ g BSA, 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM DTT, 1 mM EDTA, and 5% glycerol. DNA-protein complexes were resolved on 4% nondenaturing polyacrylamide gel electrophoresed at 12 V/cm in 0.5  $\times$  TBE. Specificity was determined by the addition of p65 or p50 antibodies (15  $\mu$ g IgG/ml; Santa Cruz Biotechnology Inc., Santa Cruz, CA) or excess unlabeled (cold) NF- $\kappa$ B oligonucleotide (20 ng) to the nuclear extracts for 10 min before addition of radiolabeled probe.

**Statistics.** Multiple comparisons were performed by one-way analysis of variance (ANOVA) and individual differences tested by the Fisher's protected least significance difference test after the demonstration of significant inter-group differences by ANOVA. Student's *t* test for

**Table I.** Lack of GSNO Effects on Various Parameters of HSVEC Viability and Protein Expression

	Control	GSNO (200 $\mu$ mol/liter, 24 h)
Cell count (cells/10 <sup>3</sup> /cm <sup>2</sup> )	55 $\pm$ 6	56 $\pm$ 8
Viability (Trypan blue exclusion)	> 95%	> 95%
Total proteins (OD mU)	60.6 $\pm$ 0.8	57.6 $\pm$ 2.1
Constitutive endothelial cell surface Ag (OD mU)		
MHC-I	825.5 $\pm$ 31.8	823.0 $\pm$ 16.7
E1/1	1726.5 $\pm$ 51.5	1745.0 $\pm$ 57.4

Values are mean $\pm$ SEM.

unpaired data was used to compare the IL-1-stimulated production of IL-6 and IL-8 with or without GSNO. Comparisons of distribution of fluorescent intensities at flow-cytometry were performed by the Kolgomorov-Smirnov's statistics (32).

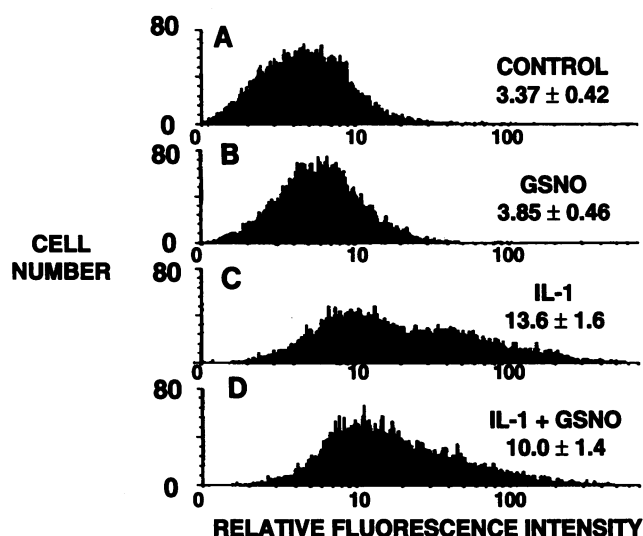
## Results

**Three structurally unrelated NO donors inhibit IL-1 $\alpha$ -stimulated VCAM-1 expression.** SNP, SIN-1, and GSNO all inhibited IL-1 $\alpha$ -stimulated expression of VCAM-1 in a dose-dependent fashion, as assessed by cell surface EIA (Fig. 1). Although maximal inhibition of adhesion molecule expression required the presence of the NO donors throughout the period of IL-1 $\alpha$  stimulation, coincubation of NO donors for periods as short as 1 h could still inhibit VCAM-1 expression, albeit to a lesser extent (see below).

Incubation of HSVEC with various NO donors for 24 h resulted in significantly different toxicity profiles, with maximum subtoxic concentrations (concentrations producing < 10% decrease in either cell count or total protein synthesis) being 10<sup>-4</sup> mol/liter for SNP and SIN-1 and 10<sup>-3</sup> mol/liter for GSNO. At 10<sup>-3</sup> mol/liter, SNP and SIN-1 significantly reduced cell number counts (12 and 14%, respectively), while GSNO did not. GSNO inhibited total protein synthesis (by 9 and 19%) only at concentrations of 1  $\times$  10<sup>-3</sup> and 10  $\times$  10<sup>-3</sup> mol/liter, respectively. Because of its lower toxicity, most subsequent experiments used GSNO.

NO itself most likely caused the inhibition of VCAM-1 expression by GSNO, since neither of its chemical precursors, nitrite and glutathione, produced any inhibitory effects at comparable concentrations (data not shown). GSNO (0.2  $\times$  10<sup>-3</sup> mol/liter) caused a 35–55% (range) reduction of IL-1 $\alpha$ -stimulated VCAM-1 expression after 24 h, but had no effect on the surface expression of two constitutive endothelial surface proteins, E1/1 and MHC-I (Table I).

Flow cytometric analysis also demonstrated inhibition of VCAM-1 expression by GSNO (Fig. 2). Unstimulated HSVEC expressed little or no VCAM-1, as the distribution of fluorescence intensity compared with cell number in such conditions (Fig. 2 A) overlapped that obtained with a control preimmune serum (not shown). Treatment with GSNO did not affect basal VCAM-1 surface expression (Fig. 2 B). Stimulation of HSVEC with IL-1 $\alpha$  shifted the cell population to the right and widened its distribution (Fig. 2 C), indicating increased VCAM-1 expression in the cell population. Compared with IL-1 $\alpha$  alone,



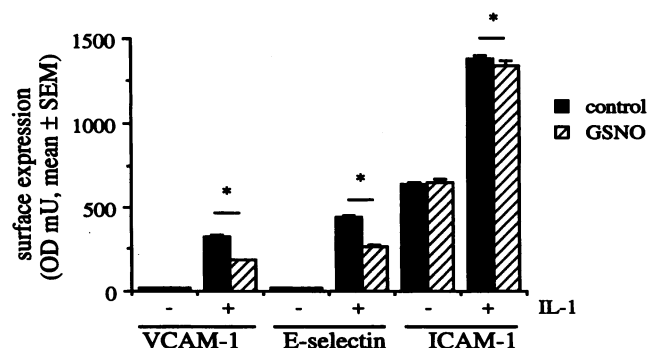
**Figure 2.** Flow cytometric analysis of endothelial cells using antibody to VCAM-1. (A) Control (no IL-1 $\alpha$ , no GSNO); (B) the effect of GSNO ( $0.2 \times 10^{-3}$  mol/liter) after 24 h; (C) the effect of IL-1 $\alpha$  (10 ng/ml) after 24 h; and (D) combination of IL-1 $\alpha$  and GSNO. Plots represent the distributions of relative fluorescence intensities (in arbitrary units, or "channels," on the abscissas, on a logarithmic scale) as a function of cell number (on the ordinates). Cell number analyzed in each condition was 10,000. Insets represent the median values of the distribution  $\pm$  SEM. Panels shown are from one of two separate experiments using different sets of HSVEC monolayers.

GSNO, when added with IL-1 $\alpha$ , shifted the distribution leftward, decreasing both its median value and its dispersion (Fig. 2D). These results indicate that, under GSNO treatment, fewer cells expressed VCAM-1 and, on the average, each cell did so to a lesser extent. Controls for flow cytometry included the use of an antibody against a constitutive and non-cytokine-inducible endothelial antigen (Ab E1/1), with which all distributions appeared uniformly shifted to the right, and a preimmune serum (results not shown), which yielded distributions similar to those shown in Fig. 2, A and B.

**GSNO inhibits other cytokine-stimulated cell-associated and secreted molecules.** We investigated the effect of GSNO on other cytokine-inducible endothelial proteins, including two other endothelial-leukocyte adhesion molecules, E-selectin and ICAM-1, and two secretable proteins, IL-6 and IL-8. GSNO inhibited cytokine-induced surface expression or secretion of each of these proteins (Fig. 3 and Table II). In comparative experiments, inhibition of inducible ICAM-1 expression, however, was negligible compared with that of either VCAM-1 or E-selectin (Fig. 3). GSNO did not affect basal (constitutive) ICAM-1 expression. Inhibition of IL-8 also was lower than for IL-6 (Table II). These experiments indicate that NO selectively decreases production of certain cytokine-induced molecules in endothelial cells.

**GSNO inhibits VCAM-1 expression in response to other cytokines.** We assessed and compared the inhibition of VCAM-1 expression by GSNO in response to a variety of structurally unrelated agonists such as IL-1 $\beta$ , IL-4, TNF $\alpha$ , and LPS. GSNO inhibited VCAM-1 expression to virtually the same extent with each stimulus tested (Fig. 4), independent of the relative potency of the stimulus.

*Maximum inhibition of VCAM-1 by GSNO requires pro-*



**Figure 3.** Comparative expression of VCAM-1, E-selectin, and ICAM-1 in HSVEC in the presence or absence of IL-1 $\alpha$  (10 ng/ml, 24 h), with or without GSNO ( $0.2 \times 10^{-3}$  mol/liter), as assessed by cell surface EIA. Data represent  $\geq 12$  replicates for each condition. \* Statistically significant differences ( $P < 0.05$ ) between groups joined by the horizontal lines.

*longed incubation.* GSNO inhibited IL-1 $\alpha$ -stimulated VCAM-1 expression after 1 h. However, the continuous presence of this NO donor during cytokine stimulation produced greater inhibition. In experiments in which exposure to the NO donor was stopped at various time points after the coaddition of the NO donor and IL-1 $\alpha$ , GSNO inhibited VCAM-1 expression by 14, 27, and 45% with 1, 6, and 24 h of coincubation, respectively. The effect of SNP and SIN-1 at the same concentrations, on the other hand, did not increase beyond the level observed after 1 h (38% inhibition).

**GSNO decreases monocyte adhesion.** Monocytes adhered little to unstimulated HSVEC (control) under nonstatic conditions at 21°C (Fig. 5, left). Exposure of HSVEC to IL-1 $\alpha$  (10 ng/ml, 24 h) increased monocyte adhesion substantially (Fig. 5, middle). Treatment of HSVEC with GSNO ( $0.2 \times 10^{-3}$  mol/liter) inhibited IL-1 $\alpha$ -induced monocyte adhesion by 61% (Fig. 5, right). IL-4 yielded qualitatively similar results (baseline:  $2 \pm 1$  cells/hpf; IL-4 50 ng/ml:  $27 \pm 5$  cells/hpf; IL-4 + GSNO:  $8 \pm 3$  cells/hpf, corresponding to a 70% inhibition). Similar effects were also observed for U937 cell adhesion (not

**Table II.** The Effect of NO (GSNO, 200  $\mu$ mol/liter, 24 h) on the Secretion of IL-6 and IL-8

	IL-6		IL-8	
	pg/ $10^5$ cells	Inhibition by GSNO	pg/ $10^5$ cells	Inhibition by GSNO
Control, no IL-1 $\alpha$	< 20		25.9 $\pm$ 3.0	
Control + GSNO	< 20	ND	25.8 $\pm$ 1.3	< 1%
IL-1 $\alpha$ (10 ng/ml)	286.0 $\pm$ 19.8		970.5 $\pm$ 31.0	
IL-1 $\alpha$ + GSNO	142.0 $\pm$ 3.3*	50%	723.1 $\pm$ 46.7*	25%

Values are mean  $\pm$  SEM of protein concentrations assayed in quadruplicate in medium from greater than four HSVEC monolayers in each control unstimulated condition and greater than eight HSVEC monolayers for each stimulated condition. Experimental conditions are similar to what is described for assay of surface-associated molecules. ND, not detectable. \*  $P < 0.01$  in comparison with corresponding control conditions.

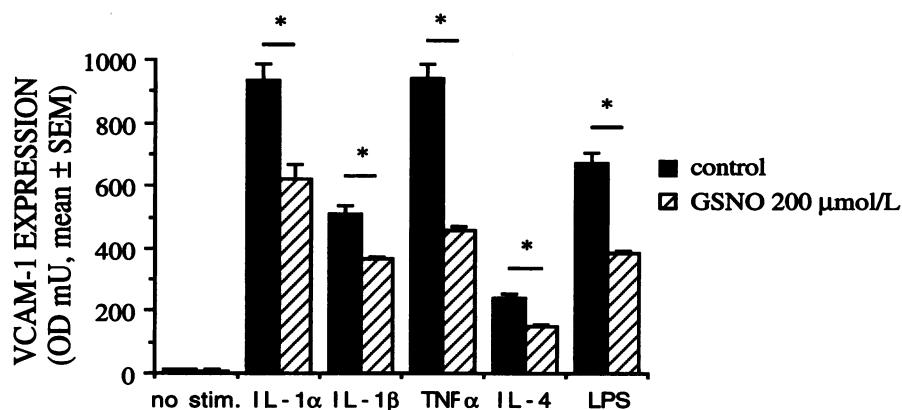


Figure 4. The inhibition by GSNO ( $0.2 \times 10^{-3}$  mol/liter for 24 h) of VCAM-1 expression by HSVEC in response to different stimuli: IL-1 $\alpha$  (10 ng/ml), IL-1 $\beta$  (10 ng/ml), TNF $\alpha$  (10 ng/ml), IL-4 (50 ng/ml), and LPS (1  $\mu$ g/ml), all administered for 24 h.  $n = 16$  for each different condition. \* Statistically significant differences ( $P < 0.05$ ) between groups joined by the horizontal lines.

shown). These observations parallel the decrease in leukocyte adhesion molecule expression caused by GSNO.

**Inhibition of VCAM-1 expression by GSNO is not mediated by cGMP.** To test whether cGMP mediates the inhibitory effect of NO donors on VCAM-1 levels, we tested the effects on IL-1 $\alpha$ -stimulated VCAM-1 expression of two membrane-permeable cGMP analogues, dibutyryl cGMP and 8-bromo cGMP, in a range of concentrations from  $10^{-8}$  to  $10^{-5}$  mol/liter. We chose these concentrations because they did not produce toxic effects on endothelial cells (as judged by morphology, Trypan blue exclusion, and cell counting) during the 24-h incubation and because they encompass concentrations able to mimic vasodilatory and antiplatelet effects of NO (3). Neither agent reduced IL-1 $\alpha$ -stimulated VCAM expression (Fig. 6 A). Furthermore, there was no trend toward any decline over the entire range of concentrations explored. Preliminary studies using metabolic labeling with [ $\gamma$ - $^{32}$ P]ATP demonstrated that 8-bromo-cGMP ( $1 \times 10^{-3}$  mol/liter) was effective in activating cGMP-dependent protein kinase activity in endothelial cells, despite lacking effects on cytokine-induced VCAM-1 expression.

**NO decreases VCAM-1 mRNA levels.** Northern analysis demonstrated decreased steady state VCAM-1 mRNA levels upon incubation with either SNP or GSNO at different time

points after IL-1 $\alpha$  addition (Fig. 6 A). Densitometric analysis of autoradiographic bands showed a 56–70% decrease compared with control levels, in good agreement with the fall in protein expression as quantified by cell surface EIA. Furthermore, GSNO ( $0.2 \times 10^{-3}$  mol/liter) diminished VCAM-1 mRNA expression over a range of IL-1 $\alpha$  concentrations (0.1–100 ng/ml) (Fig. 6 B). Inhibition of endogenous NO with L-N-monomethyl-arginine (L-NMA;  $1 \times 10^{-3}$  mol/liter) augmented the expression of VCAM-1 mRNA in unstimulated cells, but had lesser effect on VCAM-1 mRNA expression in TNF $\alpha$ -stimulated cells (Fig. 6 C). In addition, the induction of VCAM-1 mRNA expression by TNF $\alpha$  was blunted by cell-permeable PEG-catalase, and to a lesser extent, PEG-SOD, suggesting involvement of hydrogen peroxide to a greater extent than superoxide anion in TNF $\alpha$ -induced modulation of VCAM-1 mRNA (Fig. 6 D).

**NO decreases cytokine-stimulated VCAM-1 transcription.** In the presence of actinomycin D, GSNO did not significantly affect the half-life of VCAM-1 mRNA ( $5.2 \pm 1.8$  h vs.  $4.7 \pm 1.5$  h,  $P = \text{NS}$ ) indicating that the inhibitory effect of NO on VCAM-1 expression occurs at the level of transcription (Fig. 7 A). Nuclear run-on assays demonstrated a transcriptional effect of NO on VCAM-1 expression (Fig. 7 B). Transcription

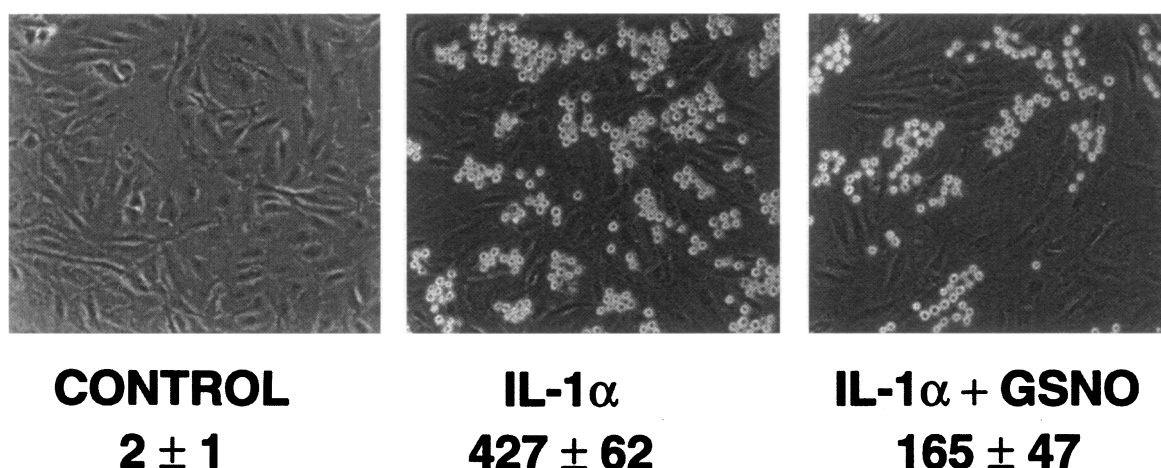
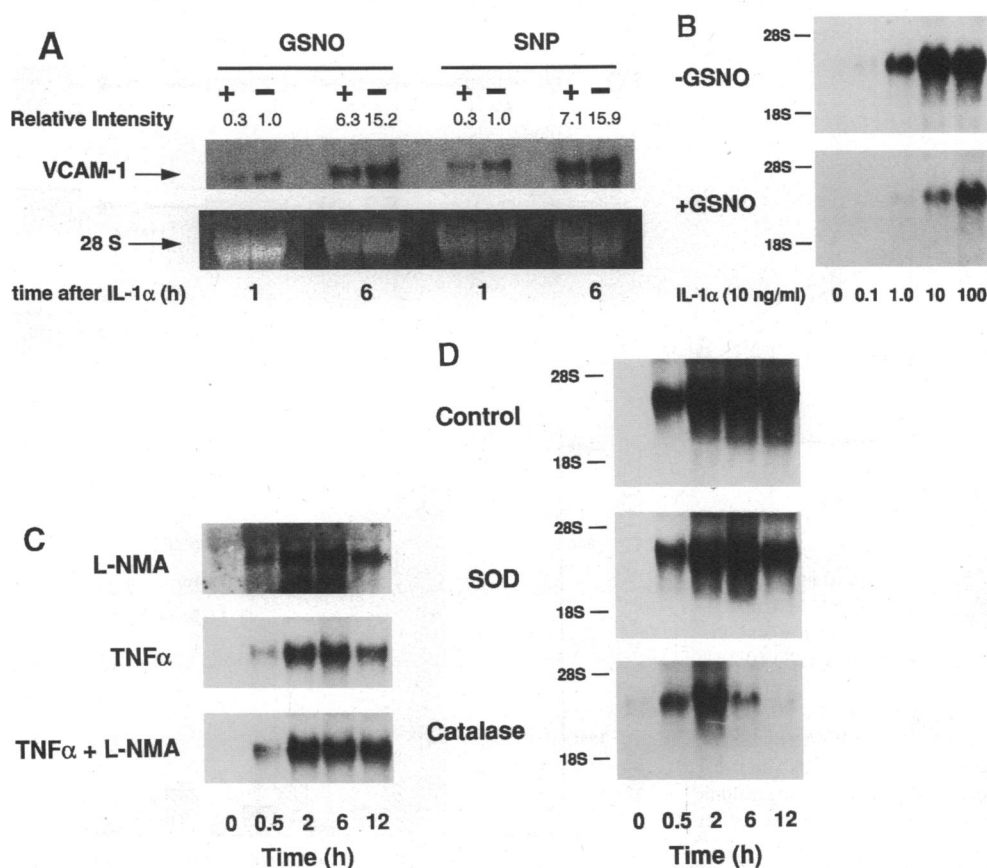


Figure 5. The inhibition by GSNO ( $0.2 \times 10^{-3}$  mol/liter for 24 h) of monocyte adhesion to HSVEC induced by IL-1 $\alpha$ , coadministered with GSNO for 24 h. Photographs represent randomly chosen fields at half-radius distance from the center of the well in one of three similar comparative experiments. Insets represent monocyte cell counts  $\pm$  SEM ( $n = 6$  for each condition) within a high-power field (magnification grid area of 0.16 mm $^2$ ).



**Figure 6.** (A) Northern analyses (20  $\mu$ g total RNA/lane) showing time-dependent effects of SNP ( $10^{-4}$  mol/liter) and GSNO ( $10^{-4}$  mol/liter) on IL-1 $\alpha$  (10 ng/ml)-induced VCAM-1 mRNA levels. Band intensities were analyzed by densitometry and expressed relative to the band intensity corresponding to IL-1 $\alpha$ -induced expression of VCAM-1 at 1 h for each set of experiments (relative intensity). The lower panel shows the corresponding ethidium bromide staining for 28 S ribosomal RNA. Three separate experiments yielded similar results. (B) Northern analyses (20  $\mu$ g total RNA/lane) showing concentration-dependent effects of IL-1 $\alpha$  on VCAM-1 mRNA expression in the presence and absence of GSNO (0.2 mM). Another independent experiment yielded similar results. (C) Northern analyses showing time-dependent effects of L-NMA (1 mM, 20  $\mu$ g total RNA/lane) or TNF $\alpha$  (10 ng/ml, 10  $\mu$ g total RNA/lane) alone, or in combination (10  $\mu$ g total RNA/lane), on VCAM-1 mRNA ex-

pression. Another independent experiment yielded similar results. (D) Northern analyses (20  $\mu$ g total RNA/lane) showing time-dependent effects of TNF $\alpha$  (Control) on VCAM-1 mRNA expression in the presence of PEG-SOD (3,000 U/ml) or PEG-catalase (40,000 U/ml). Another separate experiment yielded similar results.

of the  $\beta$ -tubulin gene served as an internal control, and lack of hybridization to the insertless vector, pGEM, established specificity (data not shown).

To localize possible sites of inhibition on the VCAM-1 promoter by NO, we performed transfection studies with various deletional promoter reporter CAT constructs (Fig. 8 A) using bovine aortic endothelial cells which yielded a higher transfection efficiency than human endothelial cells. The promoterless po.CAT and the highly expressed pSV40.CAT served as negative and positive controls in the reporter assays. The promoterless po.CAT and pF4.CAT produced essentially no relative CAT activity either basally or after stimulation with TNF $\alpha$  (Fig. 8 B). Stimulation with TNF $\alpha$  of transfectants containing the fully functional VCAM-1 promoter, F0, increased relative CAT activity from  $101 \pm 16$  to  $1,240 \pm 235$ . Cotreatment with GSNO resulted in a 76% decrease in relative CAT activity ( $300 \pm 63$ ), indicating that NO can inhibit TNF $\alpha$ -stimulated VCAM-1 promoter activity.

Transfection studies were also performed using pF3.CAT, a deletional VCAM-1 promoter construct containing two tandem NF- $\kappa$ B sites, but no AP-1 and GATA sites (Fig. 8 B). Stimulation of pF3.CAT transfectants with IL-1 $\alpha$  and TNF $\alpha$  increased relative CAT activity from  $73 \pm 23$  to  $474 \pm 52$ , suggesting that binding of NF- $\kappa$ B may be involved in the induction of VCAM-1 transcriptional activity, albeit at a significantly lower level compared with pF0.CAT. Cotreatment with GSNO

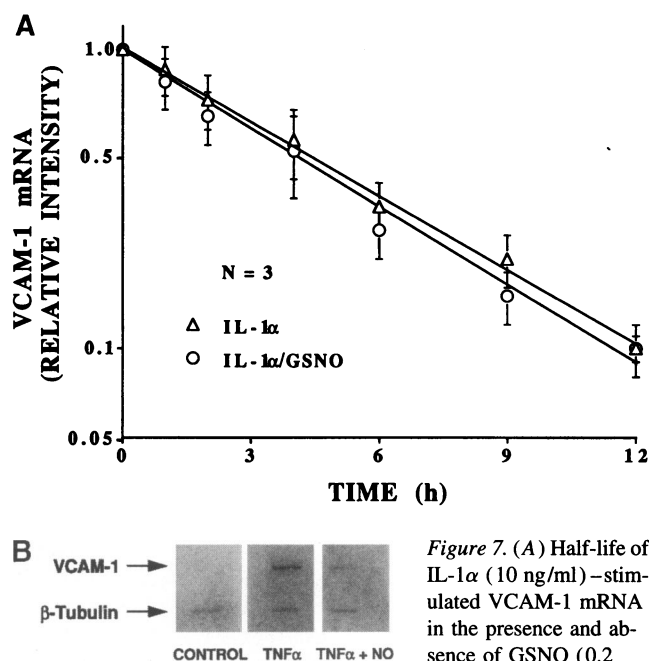
reduced the relative CAT activity of pF3.CAT transfectants to basal levels ( $139 \pm 51$ ).

**NO inhibits NF- $\kappa$ B activation.** To determine whether NO inhibits NF- $\kappa$ B activation, electrophoretic mobility shift assays were performed using radiolabeled oligonucleotide corresponding to the two tandem  $\kappa$ B sites in the VCAM-1 promoter (Fig. 9). GSNO ( $0.2 \times 10^{-3}$  mol/liter) decreased the amount of shifted complex induced by TNF $\alpha$ , indicating that NO inhibits the activation of NF- $\kappa$ B. The shifted complexes were specific for NF- $\kappa$ B since they were supershifted in the presence of antibodies to NF- $\kappa$ B subunits and disappeared with excess unlabeled oligonucleotide. In addition, neither 8-bromo-cGMP ( $1 \times 10^{-3}$  mol/liter), glutathione ( $0.2 \times 10^{-3}$  mol/liter), nor nitrite ( $0.2 \times 10^{-3}$  mol/liter) had any effects on activation of NF- $\kappa$ B (data not shown).

## Discussion

We have shown that NO can reduce cytokine-induced expression of a number of pathophysiologically relevant effector molecules characteristic of endothelial activation. Upon appropriate stimulation, the endothelium exhibits increased adhesiveness for monocytes, lymphocytes, and granulocytes. Increased or newly induced expression of endothelial-leukocyte adhesion molecules (e.g., ICAM-1, VCAM-1, and E-selectin) mediates such enhanced adhesive interactions (13, 14, 33, 34). The secre-

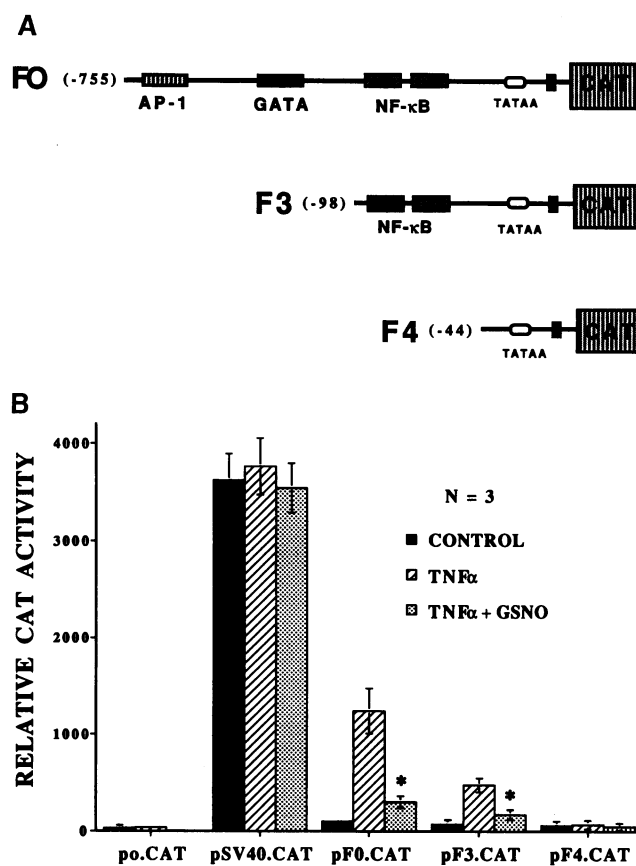




**Figure 7.** (A) Half-life of IL-1 $\alpha$  (10 ng/ml)-stimulated VCAM-1 mRNA in the presence and absence of GSNO ( $0.2 \times 10^{-3}$  mol/liter). Actinomycin D (5  $\mu$ g/ml) and GSNO were added 3.5 and 4 h after IL-1 $\alpha$  stimulation, respectively. The amount of VCAM-1 mRNA (by densitometry) was compared with the amount of VCAM-1 mRNA at 4 h after IL-1 $\alpha$  stimulation (relative intensity) and plotted as a logarithmic function of time (h). Time 0 corresponds to 4 h after IL-1 $\alpha$  stimulation. (B) Nuclear run-on assay showing the effects of NO (GSNO,  $0.2 \times 10^{-3}$  mol/liter) on TNF $\alpha$ -stimulated VCAM-1 gene transcription at 4 h. Band intensities were normalized to that of  $\beta$ -tubulin. There were no bands observed with linearized pGEM plasmid (data not shown). This is representative of two separate experiments.

tion of inflammatory cytokines (IL-6) (34) and of leukocyte-specific chemoattractants, such as IL-8 and MCP-1, also likely contributes to leukocyte recruitment during inflammatory responses in vivo (23, 35). NO significantly inhibits the expression of VCAM-1, E-selectin, and IL-6, and, to a lesser extent, IL-8 and ICAM-1. Three structurally different NO donors inhibited cytokine-induced VCAM-1 expression and concomitantly reduced monocyte adhesion to cultured endothelial monolayers. The lack of effects of nitrite or glutathione renders it unlikely that our findings were due to mediators other than NO.

The physiologic relevance of NO's inhibitory effects is supported by the finding that inhibition of endogenous NO by L-NMA can also lead to the induction of VCAM-1 expression, albeit to a lesser degree compared with cytokine activation. This result suggests that endogenous endothelial NO production tonically inhibits VCAM-1 expression in endothelial cell cultures. Because induction with cytokines results in much higher levels of VCAM-1 expression compared with L-NMA, L-NMA produced only a small augmentation of VCAM-1 expression in cytokine-induced endothelial cells. These results agree with our finding that endogenous levels of NO are not sufficient in limiting cytokine-induced VCAM-1 expression. However, at sites of inflammation, several cell types such as macrophages and vascular smooth muscle cells, have the capacity to generate 100-fold higher concentrations of inducible NO at levels comparable with the amount of NO released by GSNO in our study (36, 37). Furthermore, higher concentrations of NO could be



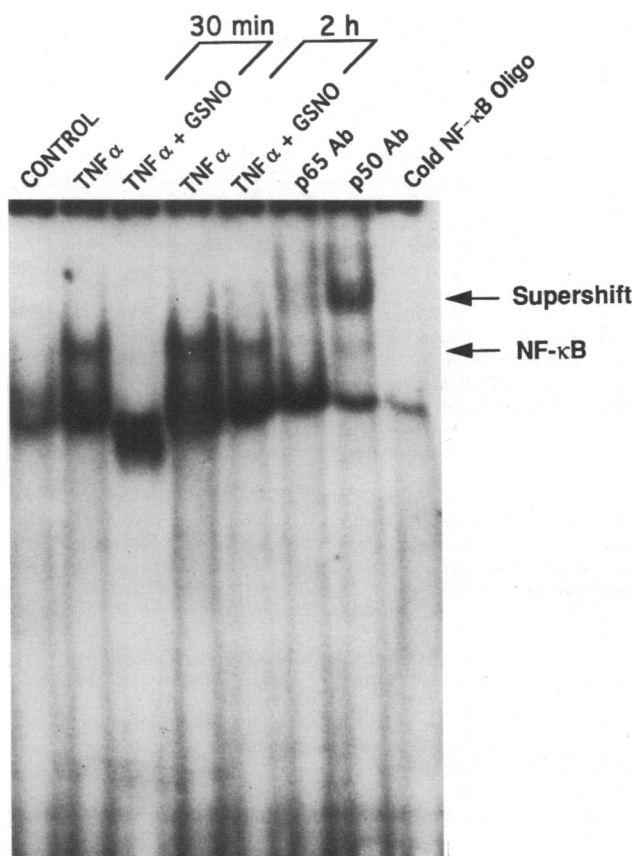
**Figure 8.** (A) VCAM-1 promoter CAT reporter constructs used in transfection experiments. The length of the promoter upstream from the initiation start site is denoted in base pairs along with putative nuclear protein binding domains. (B) Transfection studies in bovine aortic endothelial cells using promoterless construct (po.CAT), highly expressed construct (pSV40.CAT), and various truncated VCAM-1 promoter constructs. \* Significant difference ( $P < 0.01$ ) between treatment with cytokines alone and in combination with GSNO.

encountered by cytokine-activated endothelial cells due to their close proximity to smooth muscle cells and macrophages at sites of inflammation and the potential stabilization of NO by formation of potent adducts (38).

The mechanism by which NO inhibits cytokine-induced expression of VCAM-1 does not appear to involve stimulation of guanylyl cyclase since treatment with cGMP analogues does not reduce VCAM-1 expression. Nor does NO affect the post-transcriptional half-life of VCAM-1 mRNA, as demonstrated by the actinomycin D studies. Rather, nuclear run-on assays and transfection studies demonstrate that NO represses VCAM-1 gene transcription, in part, by inhibiting nuclear binding protein NF- $\kappa$ B. However, the finding that [-755]F0.CAT had a higher CAT activity in response to TNF $\alpha$  compared with [-98]F3.CAT which contained NF- $\kappa$ B sites, but not AP-1 or GATA sites, suggests that full transcriptional activity of the VCAM-1 promoter requires the participation of nuclear binding proteins such as *c-fos*, *c-jun*, and GATA.

Inhibition of [-98]F3.CAT and NF- $\kappa$ B by NO suggests that inactivation of NF- $\kappa$ B may be one of the mechanisms underlying NO's inhibitory effects on VCAM-1 expression. Indeed, other investigators have found that activation of NF- $\kappa$ B plays





**Figure 9.** Electrophoretic mobility shift assay showing the effects of GSNO ( $0.2 \times 10^{-3}$  mol/liter) on time-dependent activation of NF- $\kappa$ B by TNF $\alpha$  (10 ng/ml). Specificity was determined by addition of p65 or p50 antibodies (supershift) or unlabeled (cold) NF- $\kappa$ B oligonucleotide (20 ng) to the nuclear extracts. Three separate experiments yielded similar results.

a major role in transcriptionally inducing the expression of adhesion molecules and various inflammatory cytokines such as IL-6 and IL-8 (39, 40). Since the activation of NF- $\kappa$ B by TNF $\alpha$  is thought to occur, in part, via reactive oxygen species (41, 42), NO may inhibit NF- $\kappa$ B by scavenging and inactivating superoxide anion. The reaction of NO and superoxide anion is one of the fastest reactions known in biological systems and is almost three times faster than that between superoxide anion and SOD (43). However, at biological pH, the spontaneous dismutation of superoxide anion may be sufficiently rapid that addition of SOD may not appreciably alter the formation of hydrogen peroxide. Our findings that catalase, and to a lesser extent, SOD, inhibited cytokine-induced VCAM-1 mRNA expression agrees with reports that NF- $\kappa$ B is a more hydrogen peroxide-sensitive rather than superoxide anion-sensitive transcription factor (44).

The ability of NO to inhibit monocyte adhesion to endothelium is consistent with previous observations (7, 8). Bath and co-workers (9) reported that NO inhibits monocyte adhesion to endothelium in vitro, without altering expression of CD11b/CD18, one of the cognate ligands on monocytes for endothelial-leukocyte adhesion molecules. In experiments superfusing a cat mesenteric preparation with inhibitors of NO production (L-NMA and L-nitro-arginine methyl ester [L-NAME]), Kubes

and collaborators (7) also showed that such inhibitors increase leukocyte (mostly neutrophil) adhesion to mesenteric venules. They also showed that increased expression of CD11b/CD18 on the leukocyte surface could not account for L-NAME's effect (7). Other in vivo studies by Kurose and colleagues (45) have also documented modulation of monocyte adhesion to endothelial cells by L-NAME and reversal by cGMP analogues. This result contrasts with our finding that cGMP analogues do not affect cytokine-induced expression of VCAM-1. Several possibilities could account for this difference. First, these in vivo studies showed induction of endothelial-leukocyte adhesion by L-NAME rather than by cytokines. Perhaps induction of VCAM-1 by L-NAME is amenable to reversal by cGMP analogues. Second, these studies cannot separate cGMP effects on hemodynamics and/or leukocytes from direct alterations in endothelial functions. Furthermore, induction of other adhesion molecules (i.e., ICAM-1 or E-selectin) by L-NAME treatment may depend partially on cGMP-dependent pathways. In the present study, by selectively exposing cultured endothelial cells to NO, we have identified endothelial cells as a potential target of NO's effect in limiting leukocyte adhesion to VCAM-1.

As mentioned previously, several lines of evidence, both in vitro and in vivo, have recently suggested a role for endogenous NO as an antiatherogenic autacoid (46). Our in vitro study provides a link between NO production and monocyte adhesion and offers clues as to the mechanism of such an effect. Local generation of sufficiently high amounts of NO by either endothelial cells themselves or by other neighboring cells in response to inflammatory stimuli could provide a homeostatic regulation of monocyte adhesion under physiological conditions within the vessel wall. Endothelial dysfunction resulting in reduced level of NO activity can lead not only to an imbalance in vascular tone, favoring acute vasoconstriction (1, 2), but can also impair an endogenous negative feedback loop that limits VCAM-1 expression, leukocyte adhesion, and atherogenesis.

In summary, we have shown that exogenous as well as endogenous sources of NO can limit the degree of endothelial activation. The ability of NO to inhibit the expression of endothelial-leukocyte adhesion molecules and certain proinflammatory cytokines makes it a potentially important regulator of inflammatory trafficking within the vessel wall.

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